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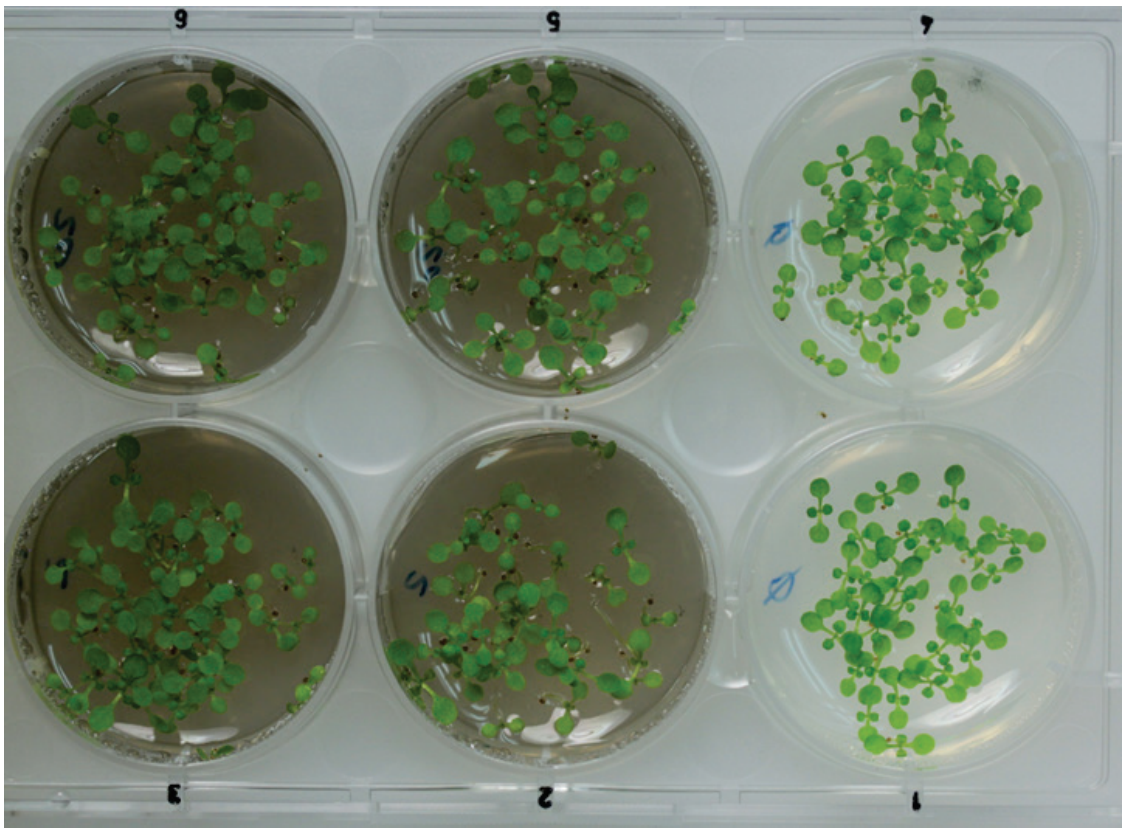
Environmental Consequences of Nanotechnologies

Bioassay to Assess Toxicity of Water-Dispersed Engineered Nanomaterials in Plants

Scientific Operating Procedure Series: Toxicology (T)

Natalie Smith, Alexander Linan, Laszlo Kovacs,
and Alan J. Kennedy

May 2019



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Environmental Laboratory
U.S. Army Engineer Research and Development Center
3909 Halls Ferry Road
Vicksburg, MS 39180-6199

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Abstract

Determining the toxicity of engineered nanomaterials (ENMs) to plants is important from an environmental perspective. Due to the absence of standardized phytotoxicity test methods for ENMs, a number of methods have been used and have produced inconsistent—and, consequently, unusable—results. This document describes a scientific operating procedure (SOP) to test the effects of plant chronic exposure to low doses of ENMs. The SOP is not specific to any particular ENM, but is designed to be applicable to any ENM that can be dispersed in water. Relying on Col-0, the highly homozygous wild-type laboratory strain of *Arabidopsis*, the method mitigates biological variance inherent in bioassay-based tests. *In vitro* growth conditions allow for the use of uniform synthetic growth medium and the exclusion of microbes. The detection of toxicity threshold is based on measuring the number of rosette leaves and the total exposed leaf area. If a physiological impact on the plant is detected, it is confirmed by the detection of ENM-induced changes in gene expression at the toxicity threshold to ensure that gene expression analysis is performed on plants grown at sublethal ENM concentrations. ENM-induced gene expression changes are then validated with the independent method of real-time quantitative PCR (qPCR).

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Preface

This scientific operating procedure (SOP) was developed Missouri State University under the coordination of the Jordan Valley Innovation Center at Springfield, Missouri. This SOP was prepared for the Headquarters, U.S. Army Corps of Engineers (HQUSACE), under the U.S. Army Engineer Research and Development Center (ERDC) and the Environmental Quality and Technology (EQT) Research Program. This SOP was used for Task 3: Environmental Health under PE 0603728A "Advancing Carbon Nanomaterials-Based Device Manufacturing through Life Cycle Analysis, Risk Assessment and Mitigation." This study was directed by Rishi J. Patel, Senior Research Scientist at Missouri State University's Jordan Valley Innovation Center. The technical monitor was Jerry Miller CEERD-EMJ.

The work was coordinated by the Environmental Risk (EPR) Branch of the Environmental Processes (EP) Division, ERDC Environmental Laboratory (EL). At the time of publication, Dr. William M. Nelson was Chief, CEERD-EPR, Mr. Jared Johnson was Acting Chief, CEERD-EP, and Dr. Elizabeth A. Ferguson, CEERD-EMJ, was the Technical Director for Military Materials in the Environment. The Deputy Director of ERDC-EL was Dr. Jack E. Davis and the Director was Dr. Ilker R. Adiguzel.

COL Ivan P. Beckman was Commander of ERDC, and Dr. David W. Pittman was the Director.

Acronyms and Abbreviations

cDNA	complementary DNA
dsDNA	double-stranded DNA
CNT	carbon nanotube
Col-0	Columbia-0 ecotype of *Arabidopsis thaliana*
dNTP	doeoxy-nucleosidetriphosphates
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
EHS	Environmental health and safety
ENM	Engineered nanomaterial
MOPS	3-Morpholinopropane-1-sulfonic acid
mRNA	messenger RNA
MS	Murashige and Skoog
NCBI	National Center for Biotechnology Information
RNA	ribonucleic acid
RNase	endonuclease that degrades RNA molecules
RPKM	reads per kilobase of exon model per million reads mapped
PPE	personal protective equipment
RNA-seq	RNA sequencing
SOP	Scientific operating procedures
TAIR	The *Arabidopsis* Information resource

Unit Conversion Factors

Multiply	By	To Obtain
mL	0.0254	millimeters

1 Introduction

The Scientific Operating Procedure (SOP) described herein for assessing the toxicity of engineered nanomaterials (ENMs) on plants was developed jointly under "The Safe and Rapid Development of Nanotechnologies Partnership and the Advanced and Additive Materials: Environmental Sustainability Research Program." The primary goal of this was to develop robust SOPs for investigating the environmental health and safety (EHS) related properties of nanomaterials.

1.1 Background

The manufacturing and use of ENMs are expected to surge in the near future (Vance et al. 2015), and the potential environmental impacts of their large-scale commercial application have raised concerns (Roco and Bainbridge 2005). Upon release or disposal, ENMs are expected to interact with environmental organisms. ENMs' interaction with plants is of interest because it is through agricultural crops that they will likely enter the food chain (Jackson et al, 2014).

Despite the foundational role of higher plants in the terrestrial food chain and several geochemical cycles, information about the effects of their chronic exposure to ENMs is scarce. Recent reports investigating the physiological impact of both metal- and carbon-based ENMs present conflicting results. Most of the conflicting results are generated by studies on the interaction of plants with carbon nanotubes (CNTs). Several investigators found that CNTs adversely affected health (Tan et al. 2009; Canas et al. 2008). Recently, Begum and Fugetsu (2012) reported that, when exposed to multi-walled CNTs, red spinach seedlings suffered toxicity associated with oxidative cell damage. In contrast, several papers have reported on the beneficial effects of CNTs on plant growth. For example, multi-walled CNTs have been found to stimulate the germination and growth of tomato (Khodakovskaya et al. 2009; Villagarcia et al. 2012) and mustard seedlings (Mondal et al. 2011), and the growth of gram plants (Tripathi et al. 2011). Multi-walled CNTs at low concentrations have been shown to improve water absorption and enhance the iron and potassium content of maize seedlings at low concentrations (Tiwari et al. 2014). Interestingly, Tiwari and co-authors (2014) registered an inhibitory effect at a higher concentration of the same multi-walled CNTs. Based on the growth

enhancement results, it has recently been proposed that CNTs and other engineered nanoparticles should be investigated for use as plantgrowth-enhancing substances in agriculture, an approach termed 'nano-biotechnology' (Srinivasan and Saraswathi 2010; Husen and Siddiqi 2014).

Data on the impact of metal-based ENMs on plant growth are more consistent than data on carbon nanostructures. Certain materials, such as aluminum oxide-, cerium oxide-, and cadmium sulfide-based ENMs, have been reported to be uniformly harmful to plants (for example, Ma et al. 2013; Marmioli et al. 2014; and Burklew et al. 2012). While the effect of metal-based ENMs is reported to be mostly adverse, results on the physiological reactions triggered by the same ENM often vary from study to study (van Aken 2015). The physiological impact of silver nanoparticles (AgNPs) on plants has been more extensively studied than that of other ENMs. AgNPs are used in numerous consumer products from which they are known to be released to the environment (Benn and Westerhoff 2008; Farkas et al. 2011), and to be ecotoxic at low concentrations (Coleman et al. 2013). Nonetheless, one study has reported an increase in growth in *Arabidopsis* when the plants were exposed to low concentrations (< 2.5 μg/mL) of AgNPs, but higher concentrations (2.5 μg/mL and above) of AgNPs uniformly lead to a reduction in biomass (Kaveh et al. 2013). Titanium oxide-based ENMs were shown to enhance photosynthesis in *Arabidopsis* (Ze et al. 2011), but to reduce germination rate and plant biomass in tobacco (Frazier et al., 2014). Therefore, a standardized procedure is needed to generate phytotoxicity data that can be comparable across various carbon-based and various metal-based ENMs.

The SOP described here facilitates the testing of chronic exposure of the model plant *Arabidopsis* to low doses of ENMs. The testing is performed under precisely controlled *in vitro* conditions, which allow the use of uniform synthetic growth medium and the exclusion of microbes, eliminating confounding effects. The detection of toxicity is based on both detecting morphological symptoms and measuring changes in gene expression at the level of the entire transcriptome. Gene expression changes are then validated using real-time quantitative polymerase chain reaction (qPCR), a standard and highly accurate method that measures relative levels of a specific mRNA. Transcriptome analysis is increasingly becoming the method to detect and characterize ENM-plant interactions (reviewed by van Aken 2015). The methodology described here was not designed to address the ecotoxicology of ENMs in complex systems such as soil.

Nonetheless, by affording precisely defined culture conditions, it is a starting point for the future development of a more robust SOP. Such a future SOP will facilitate the monitoring of the interaction between the target organism and the ENM, and the precise quantitation of the ENM in its original colloidal form and its derivatives (for example, dissolved ion, ionic derivatives, or aggregates) at the start, during the course, and at the completion of the bioassay. Meaningful approximation of the environmental fate and ecotoxicological impact of ENMs in complex natural systems is possible only if such data are obtained (Petersen et al. 2015).

1.2 Objective

This SOP is used to investigate the potential toxicity of ENMs to plants. It is applicable to any nanoparticle that can be dispersed in aqueous solutions. The scope of this SOP is limited to the initial detection of toxicity morphological symptoms on plants grown in synthetic medium under standardized axenic conditions and does not address toxicity in complex ecological systems such as soil. The characterization of the chemical behavior and the higher toxicity endpoints of ENMs also are outside the scope of this SOP.

1.3 Approach

This SOP describes a method generally applicable to test the hazard potential of ENMs that can be dispersed in an aqueous medium. The procedure utilizes the model plant *Arabidopsis thaliana* as the test organism. The SOP is performed in two phases: first, an *in vitro* bioassay is performed to determine the toxicity threshold of the ENM under study. This toxicity assay utilizes a synthetic plant medium supplemented with an ENM at various concentrations. The assay starts with growing the plants at 100 ug/ml of ENM concentration for 14 days. An ENM is considered practically non-toxic if it has no harmful effects at a concentration of 100 µg/mL or below (EPA, Technical Overview of Ecological Risk Assessment, see Related Documents). If any symptom(s) of phytotoxicity (reduced germination rate, growth retardation, chlorosis, or developmental abnormalities) are observed relative to the control plants, a range-finder assay will be performed by growing plants at decreasing concentrations of ENM to determine the toxicity threshold. Second, a transcriptome-wide gene expression study, named RNA-seq, is performed to detect differential gene expression in plants grown at the toxicity threshold. Performing RNA-seq at low doses of ENMs ensures that gene expression analysis is carried out at

sublethal concentrations. Finally, ENM-induced gene expression changes identified by RNA-seq are validated with the independent method of real-time quantitative PCR (qPCR).

RNA-seq analysis is a powerful approach, because it directly pinpoints individual genes whose expression is up- or down-regulated in response to exposure to treatment. As the molecular function and/or the biological process associated with many *Arabidopsis* genes are known, it is possible to set up meaningful and testable hypotheses for generating more information about the physiological processes affected by ENM exposure. Examining the transcriptome at a low ENM concentration brings the direct physiological impact of ENMs into focus by reducing the number of genes that are differentially expressed due to the secondary effects of stress.

2 Terminology

2.1 Related documents

- The Environmental Protection Agency: Technical Overview of Ecological Risk Assessment - Analysis Phase: Ecological Effects Characterization. Accessed on January 8, 2017 at https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/technical-overview-ecological-risk-assessment-0

2.2 Definitions

- *Arabidopsis*: A small cruciferous species, which has become the model organism for plant biologists to represent higher plants. The name of the plant is derived directly from the Latin species name *Arabidopsis thaliana*.
- Toxicity threshold: The lowest concentration at which the harmful effects of a toxic chemical are manifested as symptoms in an organism.
- Model plant: A plant that has been selected for its convenient features (ease of culturing, small size, compact genome, straightforward genetics, etc.) by the scientific community to represent similar species.
- Complementary DNA: A strand of DNA that has been synthesized based on an RNA template through the process of reverse transcription.
- Transcript: An RNA molecule that is transcribed from a gene.
- Transcriptome: The entire complement of transcripts generated from all the genes expressed in a cell, tissue, or organism.
- Stratification: The period during which water is imbibed by seeds, but germination is inhibited by low temperatures. Stratification ensures that the start of germination is synchronized in a population of seeds.
- Phenological stage: Morphologically swell-demarcated developmental stage in the lifecycle of a plant species.
- Real-time PCR instrument: A thermal cycler equipped with a mobile detector that scans the surface of the plate and detects fluorescence emitted from the reaction wells real time, as the PCR progresses.
- C_t-value: The PCR cycle at which fluorescence emitted by DNA binding dye is clearly above the level of fluorescent noise. It falls within the exponential amplification phase of the reaction and can be calculated by a software or set manually by the operator.

3 Materials and Apparatus

3.1 Materials

- Distilled water
- Agar
- 180-mL Erlenmeyer flasks
- 3-(N-morpholino)propanesulfonic (MOPS) buffer
- Potassium hydroxide (100 mM solution)
- Petri plates (Polystyrene, 100 mm x 25 mm)
- Amphotericin B
- Carbenicillin
- The ENM to be tested, dispersed in water at a known concentration
- Personal protective equipment (PPE): gloves, safety glasses, lab coat, thermo-protective gloves
- Concentrated hydrochloric acid
- Sodium hypo-chloride
- Weigh paper
- *Arabidopsis* seeds (Col-0 ecotype)
- Plastic paraffin film
- Murashige and Skoog (MS) complete medium salts (without agar, sucrose, and growth regulators)
- Liquid nitrogen
- Isopropanol
- Chloroform
- Ethanol
- DEPC-treated RNase-free water
- TRIzol
- 2.0-mL polypropylene centrifuge tubes

3.2 Apparatus

- Real-Time PCR instrument
- Temperature-controlled water bath
- Temperature-controlled water bath sonicator
- Balance
- Centrifuge for 1.5-mL Eppendorf tubes
- Environmental growth chamber
- Mortars, pestles, forceps, spatulas: for harvesting and grinding the plants for RNA extraction
- Desiccator: for seed sterilization

- Chemical fume hood
- pH meter
- Computer, requirements: 16 GB of RAM and 3 TB hard drive.

4 Procedure

The SOP is performed in three phases:

First, an *in vitro* bioassay is performed to determine the toxicity threshold of the ENM under study. This bioassay, adopted from Boyes et al. (2001) involves growing test plants under standardized axenic conditions in a synthetic medium supplemented with an ENM at various concentrations. During 14 days of growth, the number of rosette leaves and total exposed leaf area are taken as the growth rate and the vigor of the plants. If the ENM is lethal at 1 μg/mL, the experiment will be repeated at lower concentrations. If growth inhibition or another adverse effect is not detected at 32 μg/mL, the experiment will be repeated at higher concentrations of ENM. However, if the ENM triggers harmful effects at a concentration of only 100 μg/mL or higher, the ENM is considered practically non-toxic on the hazard categories provided by the EPA (Technical Overview of Ecological Risk Assessment, see in Related Documents).

Second, a transcriptome-wide gene expression study is performed to detect differential gene expression in plants grown in an ENM-containing medium. For gene expression, the *Arabidopsis* plants are grown in a medium that is supplemented with the ENM at the concentration of toxicity threshold. RNA transcripts extracted from the plants are then sequenced *en masse* in vast numbers using Illumina technology. This procedure, named RNA-seq, represents a deep sampling of the transcriptome. Transcripts that are expressed at significantly higher or lower levels relative to control are then gleaned using a bioinformatics software.

Third, ENM-induced gene expression changes are validated with the independent method of real-time quantitative PCR (qPCR).

4.1 Specimen preparation

4.1.1 Medium preparation

All components of the medium are sterilized except for the ENMs, which cannot be autoclaved or filter-sterilized without causing damage or change to their concentration. To avoid the possibility of introducing a bacterial or fungal contaminant along with the non-sterile ENMs, the medium is

supplemented with Amphotericin B and carbenicillin. The medium for each Petri plate is prepared in a separate Erlenmeyer flask. The protocol below describes the preparation of nine culture plates.

- ENMs are to be stored and ENM stocks prepared following the guidelines of the manufacturer.
- Working suspensions should be prepared by diluting stocks with sterile distilled water to the desired concentration immediately prior to their application.
- To prepare the nutrient solution, dissolve 0.225 g of MOPS buffer and 0.4875 g of MS salts in 135 mL of distilled water and adjust the pH of the solution to 7.0 with 100 mM KOH and make the volume up to 180 mL (sufficient for none plates of agar medium).
- In a sterile flask, add sterile distilled water. The amount of water and the required amount of ENM working suspension (added at a subsequent step) will make 5 mL of final volume.
- In a separate flask, add 0.2 g of agar and suspend it in 20 mL of the nutrient solution.
- Autoclave the nutrient solution/agar flask and the flask containing the distilled water at 121 °C for 20 min.
- Wearing PPE, add the desired amount of ENM working suspension to the water-containing flasks.
- Just before pouring the medium into Petri plates, pour the contents of the agar-containing flask into the ENM flask, while the water-containing flask is being held and swirled in the sonicator water bath at 55°C for 2 min. This step ensures that the nanoparticles are evenly distributed within the medium.
- Pour the contents of the flask into a Petri plate to cool and harden at room temperature.

4.1.2 Seed sterilization

- Weigh out 4.0 mg of *Arabidopsis* seeds on a sheet of weigh paper. This corresponds to approximately 100 seeds.
- Place the seeds and a beaker containing 100 mL of undiluted commercial sodium hypo-chloride solution (Clorox) into the sterilization chamber.
- Gradually add 3.0 mL of concentrated HCl in the beaker filled with bleach and close the lid of the chamber. This creates chlorine gas, which will sterilize the seeds. (This must be performed in a chemical hood).

- Leave the seeds to sterilize for 2.5 hr in the chamber.

4.1.3 Sowing, seed stratification, and plant culture

- Wearing fresh ethanol-sterilized examination gloves, pour 4 mg of *Arabidopsis* seeds onto your palm and sprinkle them evenly onto the agar medium of each Petri plate using your gloved finger and thumb.
- Seal the plate with a plastic paraffin film and incubate them in the refrigerator to stratify for three days.
- After the seeds have been stratified, take the plates out of the refrigerator and remove the paraffin film seal.
- Put each plate in its own clear, open zip-lock bag, leaving the bag unzipped. This is to prevent excessive loss of water from the medium and prevent contamination by air-borne microbes in the growth chamber. (This step may be modified by wrapping the plates with surgical tape.)
- Place the plates inside of the growth chamber with the following settings: Temperature: 20◦C, light intensity: 150 μmol/m²sec, diurnal cycle: 10 hr light, 14 hr dark.
    - Keep the plates in the growth chamber for 14 days.
    - Each day, shuffle the plates randomly within the growth chamber to avoid position effect within the chamber.

4.2 Toxicity gradient test

This data collection model was adopted from Boyes and co-authors (2001). It is a highly sensitive method that detects minor differences in the growth rate in *Arabidopsis* cultured under various treatments. The data collection model is based on determining the average number of days at which certain easily recognizable phenological stages are reached. These stages are as follows: seed imbibition (stage 0.10), radicle emergence (0.50), Cotyledon emergence (0.70), cotyledons fully open (1.0), 2 rosette leaves > 1 mm (1.02), 4 rosette leaves > 1 mm (1.04), and 6 rosette leaves > 1 mm (1.06). Growth condition described under 5.1.3 are calibrated so that the *Arabidopsis* plants will not reach beyond stage 1.06.

- Remove Petri plates with plants from the growth chamber every day until the termination of the experiment on day 14. Without removing the cover of the plates, examine the plants under a dissecting microscope. This should be done at midday every day.

- Count and record the number of plants that reached the phenological stages represented on the plates.
- At the completion of the experiment on day 14, calculate the average number of days a phenological stage is reached in each plate and the corresponding standard deviation.
- On day 14, photograph each plate alongside a metric ruler using a camera mounted on a photo stand. The camera should be directly above the plants; ensure that all plants are on the plate and the ruler included in the view field of the camera.
- Use a white background to ensure that the photo produces a plant image that is in strong contrast with the background. Use constant lighting conditions to take photos, so that images are comparable across replicates and experiments.
- Open the electronic image files in the software ImageJ (available at https://imagej.nih.gov/ij/)
- In ImageJ, use the line tool and the image of the ruler to set the scale.
- Using the Image function, adjust the color threshold by sliding brightness bars, until the plant turns red and the background white.
- Using the Analyze function, click on each plant image and then click the "ROI manager" dialog box. The software measures the visible leaf surface area of each plant, records the data, calculates summary statistics using the Result function and exports the data into an Excel sheet.

4.3 RNA-seq analysis

4.3.1 RNA extraction

- In preparation, bake mortars, pestles, forceps, and spatulas for 2 hr or more at ≥ 180 °C to deactivate any RNase contaminant. Wrap the metal-ware and mortars/pestle in aluminum foil prior to baking to ensure that they aren't RNase-contaminated after baking.
- Wearing PPE and using forceps, collect whole plants from the culture plates. The goal is to collect 75 to 80 mg of plant material (approximately 22 to 25 plants.
- As each whole plant is harvested, it is immediately submerged in liquid nitrogen in a mortar. Refill the mortar with liquid nitrogen as needed to ensure that the plants remain frozen.
- Using the pestle, grind the plants into a fine powder.

- Allow excess nitrogen to evaporate and, while the tissue is still frozen, drip 1.0 mL of TRIzol on the pulverized plant tissue, covering it as completely as possible.
    - **Note:** any steps involving TRIzol must be performed in a fume hood, because the fumes from TRIzol are toxic. The TRIzol will freeze and harden immediately into a pink-colored coating and will stay frozen for several minutes. As the mortar warms back up, the TRIzol and plant tissue will have the consistency of soft ice-cream.
- Ensure that all the plant tissue is in contact with TRIzol as it thaws by being mixed with an RNase-free spatula.
- Transfer the TRIzol-plant tissue suspension to a 2.0 mL centrifuge tube and incubate at room temperature for 5 min.
- Centrifuge at 12,000 g for 10 min at 4 °C and transfer the clear/brown supernatant to a clean 2.0-mL centrifuge tube, leaving the pellet behind.
- Add 0.2 mL of chloroform to the new tube and shake vigorously for 15 sec, then incubate at room temperature for 3 min.
- Centrifuge at 12,000 g for 15 min at 4°C, then remove the supernatant, which has the RNA, and transfer it to a fresh 2.0 mL centrifuge tube.
- Add 0.5 mL of isopropanol and mix well to precipitate the RNA and incubate at room temperature for 10 min.
- Centrifuge at 12,000 g for 10 min at 4 °C.
- Remove the supernatant and wash the pellet with 1 mL of 75% ethanol.
- Remove the ethanol and let the pellet air dry for approximately 10 min, then dissolve the pellet in 100 µL of DEPC-treated water.

4.3.2 RNA sample cleanup

This procedure is performed using RNeasy RNA-Extraction Kit (Qiagen, Cambridge, MA), following the guidelines of the manufacturer.

- Add 350 µL of RLT buffer and 250 µL of 100% ethanol to the RNA sample and mix well.
- Transfer the 700 µL mixture to an RNeasy spin column placed in a 2 mL collection tube, close the lid, and centrifuge for 15 sec at ≥ 8,000 g, then discard the flow-through.
- Add 500 µL of RPE buffer to the RNeasy spin column and centrifuge it for 15 sec at ≥ 8,000 g, discard the flow-through, then repeat this step one more time.
- Without adding more buffer to the spin column, centrifuge one more time for 1 min at ≥ 8,000 g and discard the flow-through.

- Place the RNeasy spin column in a fresh 1.5 mL collection tube and add 50 µL of DEPC-treated water directly on the spin column membrane.
- Close the lid and centrifuge for 1 minute at full speed to elute the RNA.
- Pipette the 50 µL of eluate back onto the spin-column membrane and centrifuge for a second time for 1 minute, using the same collection tube.

4.3.3mRNA purification and complementary DNA (cDNA) synthesis

Construct a complementary DNA (cDNA) library from the plant RNA using the TruSeq Stranded mRNA Sample Preparation kit by Illumina Corporation. Follow the Low Sample Size (LS) protocol of Illumina TruSeq Stranded mRNA Sample Preparation guide. The protocol will result in a library of DNA sequences that correspond to the template strand of the DNA of each gene represented by the mRNA.

The major steps of the protocol are as follows:
- Purify and fragment mRNA molecules.
- Reverse transcribe the mRNA molecules to the first cDNA strand.
- Synthesize the second strand of the cDNA using uridine nucleotides and ligate adapters.
- Degrade the second strand of cDNA and PRC amplify the remaining first strand.
- Validate, normalize, and pool the libraries.

4.3.4 cDNA sequencing

Sequencing of the libraries is to be performed on an Illumina HiSeq 2500 Sequencing System. This is done by academic or commercial genomic centers. Because the *Arabidopsis* genome is well-annotated, sequencing 100 nucleotides to form only one end of each sequence (single-end sequencing) is sufficient to unequivocally identify a gene. To obtain deep enough coverage, however, sequencing of each library is to be performed on at least two Illumina HiSeq flow cells.

4.4 qPCR Validation

The validation of RNA-seq results with an independent method is imperative. Validation ensures that false positive results are filtered out and that the data are not interpreted erroneously. qPCR technology is routinely used for RNA-seq validation. The first phase of qPCR is the

synthesis of cDNA, followed by PCR amplification of cDNA molecules derived from specific mRNA transcripts.

4.4.1 cDNA synthesis

cDNA synthesis follows the protocol adopted from the supplier of the reverse transcriptase enzyme (Invitrogen, Waltham, MA).

- Set up a reaction containing between 1 µg and 5 µg of total RNA (same RNA preparation as the one used for RNA-seq); 100 ng of random primers and 10 mM of each four dNTPs. Make up the volume to 12 µL.
- Incubate the mixture at 70°C for 5 min, then quickly transfer it to ice, and add 2 µL of 0.1 M DTT and 4 µL of 5X First-Strand Buffer (Invitrogen, Waltham, MA).
- Incubate at 25°C for 10 min, then add 200 units of SuperScript II reverse transcriptase and make up the volume to 20 µL.
- Incubate first at 25°C for 10 min, then at 42°C for 50 min, and finally, inactivate it by heating to 70°C for 15 min.

4.4.2 qPCR

The qPCR method is based on the real-time quantification of double-stranded DNA (dsDNA) present in a PCR reaction mix. The quantity of DNA is measured as the amount of fluorescence produced by a dye (SYBR Green) that emits light only when it is bound to dsDNA, the amount of which is commensurate with the PCR product. qPCR experiments are required to be performed in a real-time PCR instrument.

- Set up 20 µL reactions in a 96-well PCR plate using the GoTaq qPCR Master Mix (Promega Corp., Madison, WI)
- In each 20-µL reaction, combine 10 µL of GoTaq qPCR Master Mix, 50nM of CXR reference dye, 0.2µM of primers, and 150 ng of cDNA.
- For each primer pair, prepare a no-template control in which the cDNA is replaced with water.
- Cover the wells with a transparent cover, and run the reactions in a real-time PCR instrument with the flowing thermal profile: (1) one pre-melt cycle at 95◦C for 2 min, (2) 40 cycles at 95◦C for 15 seconds and 60◦C for 1 minute, and (3) 1 dissociation cycle at 95◦C for 15 sec, 50◦C for 30 sec and 95◦C for 30 sec.
- Each reaction is replicated in three wells, and the entire experiment is repeated for two technical replicates.

- Following the completion of the experiment, set the SYBR Green detection threshold (C_t-value) to the lab standard, and record the C_t-value for each reaction.

5 Reporting

5.1 Analysis of results:

5.1.1 Toxicity gradient test

5.1.1.1 Growth Rate

- For control and each treatment, record the day in which each of the 7 growth stages appear for each plant. Compare the average day for each growth stage between treatments and the control using one-way ANOVA in statistical software such as MiniTab or S+.
- Make sure to preform ANOVA on each treatment at each growth stage individually against the control.
- A difference in growth rate is considered significant at a *p*-value of 0.05 or lower.

5.1.1.2 Exposed Leaf Area

- Compare the exposed leaf area between treatments and the control using one-way ANOVA in statistical software such as MiniTab or S+.
- Make sure to preform ANOVA on each treatment individually against the control.
- A difference in growth rate is considered significant at a *p*-value of 0.05 or lower.

5.1.2 RNA-seq analysis

To identify genes that are differentially expressed in response to exposure to nanoparticles, RNA-seq differential expression analysis is to be performed. This analysis is to be carried out using a bioinformatics software package which provides all algorithms for the (1) alignment of overlapping sequence reads, (2) alignment of the resulting contigs to the *Arabidopsis* reference genome, (3) annotation of the transcripts, (4) quantification of the expression level of each *Arabidopsis* gene, and the (5) statistical analysis of differences in expression between treatments. The instructions below are described using the example of the commercially available software suite CLC Genomics Workbench 9.5.1. (Qiagen, Cambridge, MA). The list of genes identified as responsive to ENMs may contain false positives. To glean the most dramatically regulated genes and eliminate most (and likely all) false positives from the extensive list, perform a Bonferroni or false

discovery rate correction and limit the list of genes to those that are up- or down-regulated 3-fold or higher.

- Download raw RNA-seq sequence read data form the server of the sequencing center via a SFTP client using a SSH File Transfer Protocol.
- Combine RNA-seq data into a single FASTQ file for each biological replicate.
- Load the zipped concatenated FASTQ files as Illumina files into the database of CLC Genomics Workbench 9.5.1.
- In CLC Genomics Workbench, unzip the FASTQ files.
- Trim RNA sequences to have a minimum quality of 0.05.
- Remove 15 base pairs from the 5' (prime) terminal end of each sequence.
- This is done to remove the adaptors that were added during the cDNA library construction.
- Filter sequences using the default parameters.
- Map the concatenated trimmed sequences against the reference genome of *Arabidopsis thaliana* (available at the National Center for Biotechnology Information [NCBI] and *Arabidopsis* Information Resource [TAIR]).
- Determine the expression level of each gene in each sample by calculating reads per kilobases of exon model per million mapped reads (RPKM).
- Use default parameters except for the strand specification, which is to be set for Forward.
- Perform statistical analysis using multi-group paired comparison. This is the appropriate statistical approach, as the biological replicates (repeats) of the various treatments are linked (because they were performed parallel).
- When calculating significance, apply Bonferroni correction or false discovery rate to control the number of genes that are false positives (Type I error) for responding to ENMs.
- To narrow the gene list to most dramatically regulated genes, consider only those that are up- or down-regulated 5-fold or higher.

5.1.3 qPCR validation

The purpose of the qPCR validation is two-fold: first, it is to determine how many times a gene in the ENM-treated samples is up- or down-regulated relative to the control; second, it is to determine whether the up- or down-regulation of a gene is significant. Because PCR amplification is

the function of both the DNA abundance and the efficiency of the PCR fold change, calculations must be carried out using the Pfaffl equation. The fold-change values are normalized by measuring the expression level of a reference gene in both the treated and the control samples. The gene Actin-2 (*ACT-2*) is used as a reference because it is thought to have a constant level of expression over a broad range of conditions. The measurements confirmed the stability of *ACT-2* expression (see Table 3 and Fig. 2 in Notes and Supplementary Data).

5.1.3.1 Fold-change of gene expression relative to control

- To analyze the qPCR data, first the C_t values from the two technical replicates are averaged.
- Calculate gene expression fold-change using the Pfaffl equation:

$$Fold\ change\ relative\ to\ control = \frac{(E_{target})^{\ \Delta Ct\ target\ (control-treated)}}{(E_{reference})^{\ \Delta Ct\ reference\ (control-treated)}}$$

Where E represents the amplification efficiency of the target DNA. The value of E has been determined for 13 genes that significantly changed in expression, plus the reference gene *ACT2,*

and is shown in Table 1 in the Annex at the end of this document.

5.1.3.2 Statistical significance of change in gene expression

- Subtract the average C_t value for the target gene from the average C_t value for the reference gene.
- Then analyze the resulting *delta-*C_t values using one-way ANOVA in the statistical software such as Minitab or S+.
- Consider a p-value of <0.05 to indicate significance.

5.2 Key results provided

5.2.1 Toxicity gradient test

Provide a table detailing the average day that plants reached each growth stage for each treatment. In a separate table, provide exposed leaf area data for each treatment. Also, include in both tables standard deviation between plant replicates as well as coefficient of variation.

Results provided from the one-way ANOVA test should include at least the Analysis of Variance table, which contains the *p*-value. Other information that can be provided includes method information, factor information, model summary, and table of means. For visual representation of data, the interval plot between treatment (concentration) and control can be provided.

RNA-seq Analysis

Provide a list of genes that were found significantly differentially expressed in ENM-exposed and control plants after multi-group paired comparison analysis and Bonferroni correction.

For each gene, provide the expression value (in RPKM) for the control plants, the treated plants, and the fold-change of up- or down-regulation. If the molecular function or biological role of the gene's protein product is known, provide that information also. (See Table 2 in Notes and Supplementary Data).

Determine in which molecular function, biological process, or cellular component categories the list of regulated genes is enriched. Provide the significance level of this enrichment (see Fig. 1 in in Notes and Supplementary Data). Additional gene expression data visualization tools are available for *Arabidopsis* at the web resources ePlant (bar.utoronto.ca) and the TAIR (www.arabidopsis.org).

In addition, metadata that characterize the sequencing study, including the number of raw reads obtained for each replicate, the average read length, the number of reads mapped, the number of transcripts identified, and the number of transcripts represented by reads > 10 should also be reported.

qPCR validation

Provide a table listing the reference and target gene efficiencies that were used during qPCR (Table 1A in Appendix A).

Provide a table summarizing results between RNA-seq and qPCR for easy comparison. This table should include fold change and *p*-values from RNA-seq analysis, and the fold change calculated from qPCR Pfaffl

Equation and the one-way ANOVA *p*-value (see Table 3 in Notes and Supplementary Data).

Results provided from the one-way ANOVA test should include at least the Analysis of Variance table, which contains the *p*-value. Other information that can be provided includes the ANOVA method, factor information, model summary, and table of means. For visual representation of data, the interval plot between treatment (concentration) and control can be provided.

Quality Assurance/Quality Control Considerations

To ensure consistency of the bioassay, the *Arabidopsis* Col-0 seeds must be obtained from a source that guarantees that the Col-0 ecotype seed are true to type. There are three such sources: The Arabidopsis Biological Resource Center at Ohio State University and Lehle Seeds (Round Rock, Texas) in the U.S., and the Nottingham Arabidopsis Stock Centre at the University of Nottingham in the UK. When grown on control medium under experimental conditions, the seed germination rate must be at least 90%, and most plants should reach phenological stage 1.04, but not beyond 1.06. Seed quality is unacceptable if the control germination rate is below 90%, and most plants are below stage 1.04 by the end of 14 days' culture.

This SOP assumes that the ENM under study will not change its initial concentration beyond +/-20% during the experiment. Based on the Organization for Economic Cooperation and Development guidelines, the meaningful interpretation of the results will require that the 20% exposure specification is maintained (Petersen et al. 2015). While medium preparation protocol in this SOP is designed to minimize the chemical transformation and the aggregation of ENMs, the SOP in its current state does not provide data on the consistency of ENM exposure during the course of an experiment.

In RNA-seq analysis, it is essential that at least 95% of the reads (contigs) are aligned to the reference genome. Lower percentage of read mapping indicates experimental or operational error, and such data are unacceptable for further analysis. As the *Arabidopsis* genome is well mapped and annotated, the alignment of 95% of the reads can be readily accomplished. In the pilot experiment with Ag QDs, the team was able to meet this requirement.

In RNA-seq analysis, *Arabidopsis* genes that have an RPKM value of < 10 should be excluded from expression-level analysis. Low-level expressed genes have a high probability to have false differential expression due to chance playing a much greater role in read count (high measurement error).

Conclusions

This SOP tests the hazard potential of water-dispersed ENMs in plants through measuring growth rate and gene expression. To minimize both environmental and biological variance, the SOP is based on a bioassay performed under *in vitro* conditions on a standard laboratory line of the model organism *Arabidopsis thaliana*. The bioassay facilitates the chronic exposure of the plant throughout its development from embryo to four-rosette-leaf stage to ENMs at concentrations below the toxicity threshold. The toxicity threshold of an ENM is determined with a range-finder assay which involves growing plants at 100 µg/mL and gradually decreasing concentrations. The SOP specifies that gene expression measurements are made sequentially with two independent methods: first, with a genome-scale RNA-seq analysis; second with qPCR which confirms the expression levels of selected differentially regulated genes. Thereby, validation of the results is built into the SOP. The advantage of characterizing the impact of ENMs in terms of both growth rate and a validated gene expression signature is that the data can point to meaningful and testable hypotheses.

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Appendix: Notes and Supplementary Data

Table A1. Efficiency values for 14 genes used in our qPCR analysis to validate expression level changes

Gene	Efficiency
ACT2	1.976
AZI1	1.966
RAP2.3	1.893
AT3G16670	1.951
MIOX4	1.881
ACD6	1.882
GRP5	1.944
CHIB	1.987
GASA6	1.871
PCC1	1.884
PCR1	2.005
AT1G77330	1.831
AT5G03350	1.814
WAK1	1.894

Table A2. List of 36 Arabidopsis genes that were significantly up- or down-regulated at Bonferroni corrected $p<0.05$ in response to Ag QD exposure. Total number of genes identified by RNA-seq as responsive to Ag QDs at $p<0.05$ were 674; the number of genes that were responsive to Ag QDs at FDR-corrected $p<0.05$ were 81.

Gene Name	Fold-Change in Expression	Control - Expression Values (RPKM)	Ag QD - Expression Values (RPKM)	Function
NUDT6	12.73	1.53	22.17	Encodes a protein with NADH pyrophosphatase activity; involved in positive regulation of salicylic acid-mediated signaling pathway.
AIR1	12.08	2.61	34.58	Encodes a protein that is related to a large family of proteins that have a hydrophobic, possibly membrane spanning C-terminus. Responsive to auxin. Carbohydrate transmembrane transporter activity, sugar:hydrogen symporter
AT5G24200	10.67	1.07	13.35	Alpha/beta-Hydrolases superfamily protein; functions in triglyceride lipase activity.

Gene Name	Fold-Change in Expression	Control - Expression Values (RPKM)	Ag QD - Expression Values (RPKM)	Function
AT5G18840	9.68	2.67	28.54	Carbohydrate transmembrane transporter activity, sugar:hydrogen symporter
AT5G10760	8.37	3.04	28.12	Aspartic-type endopeptidase activity
AT5G03350	6.94	8.58	65.13	Legume lectin family protein; involved in cellular response to salicylic acid stimulus, phosphorylation, systemic acquired resistance.
ACD6	6.28	8.27	57.56	Accelerated cell death 6, apoptosis regulator, responds to salicylic acid stimulus and defense response to pathogens
MIOX4	6.27	19.04	117.98	Encodes a myo-inositol oxygenase, involved in L-ascorbic acid biosynthetic process, inositol catabolic process.
TPPG	5.46	5.85	34.58	Haloacid dehalogenase-like hydrolase (HAD) super-family protein; functions in trehalose biosynthesis.
CPuORF27	5.44	5.83	34.33	Upstream open reading frames (uorfs) in the 5' UTR of a mature mRNA, and can potentially mediate translational regulation of the major, ORF (morf).
CML47	4.89	4.28	23.10	Unknown protein
WAK1	4.36	6.31	30.49	Cell-wall associated kinase that functions as cell signaling receptor for oligogalacturonides.
AT2G44670	3.17	50.46	171.71	Senescence-associated family protein
AT4G16146	-3.52	46.69	14.37	cAMP-regulated phosphoprotein
EBF2	-3.92	73.37	20.03	Protein involved in the ethylene-response pathway.
ERF113	-4.04	23.61	6.07	Unknown protein
UPB1	-4.48	14.60	3.38	Regulates the expression of peroxidases that modulate reactive oxygen species. Involved in differentiation.
AT4G16260	-4.80	65.12	13.92	Plays a role in host defense.
AZI1	-4.94	249.47	57.04	Priming of salicylic acid induction and systematic immunity by pathogen.
UGT75D1	-5.04	21.29	4.60	Unknown protein
AT1G77330	-5.31	37.24	7.29	1-aminocyclopropane-1-carboxylate oxidase activity, involved in ethylene synthesis

Gene Name	Fold-Change in Expression	Control - Expression Values (RPKM)	Ag QD - Expression Values (RPKM)	Function
AT2G43590	-5.40	62.93	12.61	Chitin binding, carbohydrate metabolic process, cell wall macromolecule catabolic process
AT1G73120	-5.49	62.12	11.44	Responsive to oxidative stress
AT5G01210	-5.90	43.59	7.81	Regulation linked to pathogen response, abscisic acid and ethylene signaling
AT1G26250	-6.65	37.97	5.96	The gene product is a structural constituent of cell wall.
PDF2.5	-6.69	16.81	2.59	Involved in defense response, defense response to fungal attack.
AT3G54040	-6.78	38.75	5.98	Signal transduction by ethylene, a stress hormone in plants.
UGT74E2	-7.30	26.35	3.76	Encodes a UDP-glucosyltransferase, UGT74E2, that acts on IBA (indole-3-butyric acid) and affects auxin homeostasis.
AT3G16670	-10.17	76.54	7.70	Responsive to oxidative stress.
ARL	-10.30	16.37	1.57	Protection of plants from various types of stresses including salts and heavy metals.
AT5G02760	-13.42	10.01	0.68	Encodes a phosphatase that functions in sustaining proper leaf longevity and preventing early senescence by suppressing or perturbing SARK-mediated senescence signal transduction
AT2G05510	-13.91	478.30	36.66	Unknown protein
RAP2-3	-23.35	295.56	13.42	Ethylene response factor (ERF). A key regulatory hub in plant responses to abiotic stresses.
ATNAC2_2	-26.44	31.38	1.16	Plant-specific transcription factor
PER59	-136.30	18.38	0.02	Peroxidase
PER10	-195.63	25.09	0.01	Peroxidase

Table A3. Fold-change values for reference gene *ACT2* and for thirteen Arabidopsis genes regulated by exposure to Ag QDs (at 4 µg/mL) as determined by RNA-seq and qPCR. The statistical significance (*p*-value) of expression level difference in control and in Ag QD-exposed plants also is shown. For RNA-seq data, FDR- and Bonferroni-corrected *p*-values also are shown. These 13 genes were chosen based on fold-change and expression level from 36 genes with Bonferroni corrected $p<0.05$ (see Table 2).

	RNA-seq				qPCR	
Gene	Fold Change	*p*-value	FDR-corrected *p*-value	Bonferroni-corrected *p*-value	Fold Change	*p*-value
ACT2	1.206	0.331	1	1	1	
AZI1	-4.936	5.61E-07	0.0006	0.019	0.210	0.069
RAP2.3	-23.346	3.35E-22	5.63E-18	1.13E-17	0.045	0.000
AT3G16670	-10.171	6.9E-14	2.9E-10	2.32E-09	0.071	0.003
MIOX4	6.268	1.168E-07	0.0002	0.004	3.861	0.021
ACD6	6.280	3.143E-07	0.0004	0.011	4.388	0.006
GRP5	4.137	0.0002	0.066	1	1.985	0.236
CHI-B	-3.529	0.0001	0.053	1	0.323	0.035
GASA6	-4.588	5.092E-05	0.025	1	0.171	0.049
PCC1	10.942	8.817E-06	0.006	0.296	13.937	0.024
PCR1	8.058	4.09E-05	0.023	1	6.021	0.104
AT1G77330	-5.314	6.630E-09	1.238E-05	0.0002	0.170	0.013
AT5G03350	6.940	1.064E-07	0.0001	0.004	1.339	0.847
WAK1	4.364	1.118E-06	0.001	0.038	2.802	0.031

Figure A1. Relative frequency of 81 Ag QD-responsive (FDR corrected p<0.05) genes classified by biological processes, molecular function, and cellular component. Bold letters indicate that the Ag QD-responsive gene list is significantly enriched (p<0.05) in the categories. Image created with Classification SuperViewer with Bootstrap (Provart & Zhu, 2003).

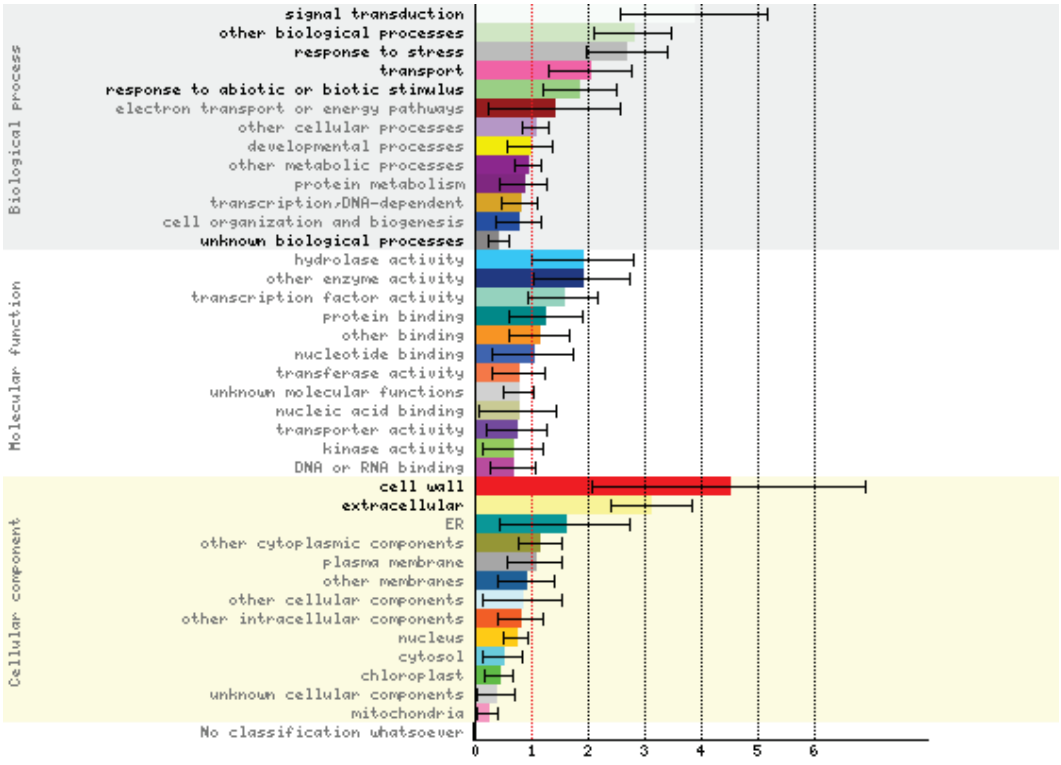
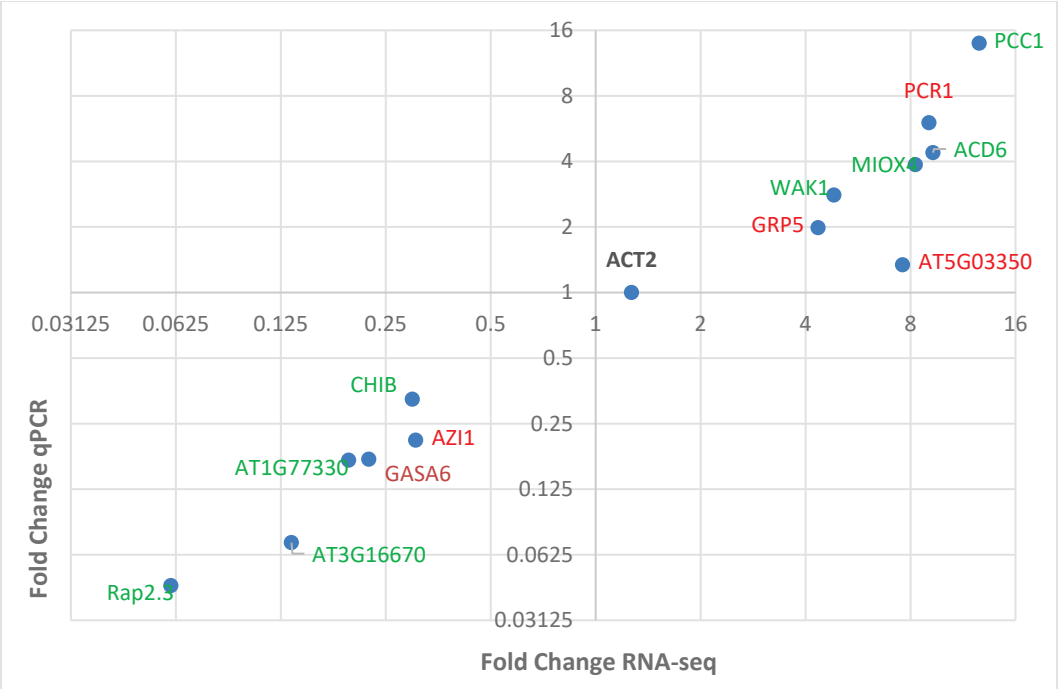


Figure A2. Change in expression level of the reference gene *ACT2* and thirteen Arabidopsis genes regulated by exposure to Ag QDs (at 4 µg/mL) as determined by RNA-seq and qPCR. The RNA-seq- and qPCR-measured fold-change values are plotted against one-another to demonstrate correlation between measurements.



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14. ABSTRACT

Determining the toxicity of engineered nanomaterials (ENMs) to plants is important from an environmental perspective. Due to the absence of standardized phytotoxicity test methods for ENMs, a number of methods have been used and have produced inconsistent—and, consequently, unusable—results. This document describes a scientific operating procedure (SOP) to test the effects of plant chronic exposure to low doses of ENMs. The SOP is not specific to any particular ENM, but is designed to be applicable to any ENM that can be dispersed in water. Relying on Col-0, the highly homozygous wild-type laboratory strain of *Arabidopsis*, the method mitigates biological variance inherent in bioassay-based tests. *In vitro* growth conditions allow for the use of uniform synthetic growth medium and the exclusion of microbes. The detection of toxicity threshold is based on measuring the number of rosette leaves and the total exposed leaf area. If a physiological impact on the plant is detected, it is confirmed by the detection of ENM-induced changes in gene expression at the toxicity threshold to ensure that gene expression analysis is performed on plants grown at sublethal ENM concentrations. ENM-induced gene expression changes are then validated with the independent method of real-time quantitative PCR (qPCR).

15. SUBJECT TERMS
bioassay, carbon nanomaterials, engineered nanomaterial toxicity, ERDC NanoGRID, Filmetrics Thin Film Analyzer, life cycle analysis, nanomaterial film, nanotechnologies, refractive index, spectral reflectance, thin film thickness, toxicology

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